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Effects of the deficiency of the rhodanese-like protein RhdA in *Azotobacter vinelandii*

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Abstract **In *Azotobacter vinelandii* the *rhdA* gene codes for a protein (RhdA) of the rhodanese-homology superfamily. By combining proteomics, enzymic profiles and ultrastructural observations, the phenotype of an *A. vinelandii rhdA* mutant was analyzed. We found that the *A. vinelandii rhdA* mutant, and not the wild-type strain, accumulated polyhydroxybutyrate. RhdA deficiency enhanced the expression of enzymes of the polyhydroxybutyrate biosynthetic operon, and affected the activity of specific tricarboxylic acid cycle enzymes. The effect was dramatic on aconitase, in spite of comparable expression of aconitase polypeptides in both strains. By using a model system, we found that RhdA triggered protection from oxidants.**

Keywords: Rhodanese; *rhdA* mutant; Protein profiles; Polyhydroxybutyrate; *Azotobacter vinelandii*

1. Introduction

Proteins displaying sequence homology with bovine rhodanese (thiosulfate:cyanide sulfurtransferase, [1]) are widespread in all major evolutionary phyla. In the rhodanese homology superfamily (Accession number: PF00581; http://sanger.ac.uk/cgi-bin/Pfam), the rhodanese domains are present as single or tandem repeats, and also as modules in multidomain proteins [2]. The biological role of rhodaneses is still largely debated, and the finding that most organisms have multiple rhodanese homology proteins, makes it likely that they could serve with some specificity in particular cellular pathways. Proposed functions for rhodaneses include cyanide detoxification, formation of prosthetic groups in iron–sulfur cluster proteins, and sulfur transfer for thiamine and thiouridine synthesis [3–6]. Physiological roles for rhodanese homology domain were identified in the biosynthesis of 4-thiouridine in tRNA [6], in selenium transfer from selenophosphate during conversion of 2-thiouridine to 2-selenouridine in bacterial tRNA [7], and in the pathway for the biosynthesis of molybdenum cofactor in humans [8].

The variety of pathways in sulfur metabolism, and the peculiar role of inter-protein recognition in these events, makes it interesting the investigation on biological targets of specific rhodanese homology protein.

The two-domain rhodanese protein RhdA from *Azotobacter vinelandii* [9] shows some peculiar properties in the panorama of the rhodanese-related enzymes. The RhdA active-site motif (HCQTHHR) is not currently found in rhodanese-like proteins, and it appears properly designed to support stabilization of a persulfide bond on its Cys_{230} catalytic residue [10,11]. Recently, we demonstrated that the stable sulfane-sulfur-bearing form of RhdA (RhdA-SSH) was generated via reaction with the cysteine desulfurase IscS [12], and that RhdA-SSH can function as sole sulfur source for *in vitro* reconstitution of Fe–S clusters [13].

In the present report, to investigate *in vivo* functions of RhdA, we characterized, by combining proteomics, enzymic profiles and ultrastructural observations, an *A. vinelandii* mutant strain (MV474) in which the *rhdA* gene was disrupted by deletion [9]. In the *rhdA* mutant, and not in the wild-type *A. vinelandii* UW136, the expression of the enzymes β-ketothiolase and acetoacetyl CoA reductase, coded by the *phbA* and *phbB* genes present in the polyhydroxybutyrate (PHB) biosynthetic operon [14], was enhanced giving raise to accumulation of PHB. Furthermore, RhdA deficiency affected the activity of Fe–S enzymes of the tricarboxylic acid cycle (TCA), and protein profile comparisons provided evidences that the dramatic effect on aconitase was related to the production of inactive enzyme. A model system was, therefore, exploited to analyze whether RhdA could have a role in protecting cells from oxidative damage.

2. Materials and methods

2.1. Bacterial strains, cell culture, and treatments

The *A. vinelandii* strains used in this study were UW136 and a derivative of UW136 (MV474) in which disruption of the *rhdA* gene was achieved by insertion of a KIXX cassette, following deletion of 584 bp as described in Ref. [9]. In MV474 the KIXX cassette insertion was with orientation that gives transcription and expression of downstream genes [Ref. [9] and R. Colnaghi, unpublished].

Cells were grown aerobically in Burk's medium BSN for 25 h at 30 °C supplemented with 1% sucrose as carbon source. When the

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optical density reached $OD_{600} = 2.8$, the cells were spun down at $3800 \times g$ for 10 min, and stored at −80 °C. Polyhydroxybutyrate (PHB) content was determined by a spectrophotometric method [15]. For ultrastructural observations, the bacteria cultures were washed with phosphate buffer (PB) 0.1 M, pH 7.2, and fixed overnight in a mixture of 3% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde in the same buffer. After thoroughly washing for 1 h with PB, they were post-fixed for 2 h in 1% osmium tetroxide in PB. Samples were dehydrated in an ethanol series and embedded in Spurr's resin. Ultrathin sections were stained with 2% aqueous uranyl acetate and lead citrate, and examined in a Jeol 100SX transmission electron microscope (TEM, Jeol, Japan).

To investigate the effect of the oxidative agent phenazine methosulfate (PMS), cells were grown in Burk's medium BSN at 30 °C supplemented with 1% sucrose up to $OD_{600} = 0.800$, then the cultures of either UW136 or MV474 strains were divided into two equal samples one of which was treated with PMS (final concentration 15 μM).

2.2. Sample preparation and 2-DE

Cell cultures were centrifuged (30 min at $2000 \times g$), and the pellets resuspended in 10 mM Tris–HCl, pH 8.0 buffer containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% SDS, and stored at −20 °C. Before analysis, 100 μL of sample were solubilized in 1 mL of lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM dithiothreitol (DTT), 2% IPG buffer pH 4–7 (Amersham Biosciences)), and incubated for 20 min in ice. Protein concentrations were estimated using 2-DE quant Kit (Amersham Biosciences) before adding bromophenol blue. For 2-DE analysis, 180 μg of proteins were loaded onto Immobiline DryStrips (pH 4–7 linear, 14 cm long; Amersham Biosciences), and the overnight in-gel reswelling method (Amersham Biosciences Instruction Manual) was used. IEF was carried out at 20 °C using a Multiphor II electrophoresis unit (Amersham Biosciences) with the following program: 75 V for 1 h, 300 V for 1 h, 300–1000 V in 4 h, 1000–8000 V in 6 h, 8000 V for 2 h, and 50 V for 1 h. After focusing, Immobiline Dry-Strips were equilibrated for 15 min at room temperature in the equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS) containing 1% w/v DTT, followed by a second incubation step (15 min) in the equilibration buffer in the presence of 2.5% w/v iodoacetamide. Separation in the second dimension (SDS–PAGE) was carried out on homogeneous 12% T gels, and electrophoresis was conducted at 10 mA per gel for 30 min, and at 25 mA for 4 h in a Hoefer SE600 Electrophoresis Unit. Gels were stained with Coomassie Brillant Blue G-250. In order to corroborate the reproducibility of the results, at least three extractions were made for each strain, and three gels were run for each sample. Immunological detection of RhdA protein blotted from 2-DE gels on to nitrocellulose membranes was performed as previously described [13], by using selective anti-RhdA antibodies [16].

2.3. Image acquisition and analysis of 2-DE gels

Blue Coomassie stained gels were digitized using an EPSON Expression 1680 Pro scanner (Epson, Milan, Italy) and analyzed with the ImageMaster 2D Platinum software (Amersham Biosciences). Gel patterns from independent analyses were matched together, and the relative abundance of each spot (%V) in the two gel sets (UW 136 and MV474 *A. vinelandii* strains) were compared. The %V represents the pixel density of each spot normalized for the total pixel density from all the spots in the same gel.

2.4. MS analysis of protein spots and protein identification

In gel enzymatic digestion of the spots and peptide extraction were carried out as described in Ref. [17]. MALDI-TOF MS analysis was performed in a Voyager DE-Pro spectrometer (PerSeptive BioSystems,Framingham, MA). External mass calibration was performed with low-mass peptide standards and mass measurement accuracy was ±0.3 Da. The mass spectra were acquired in the reflector mode using the Delay Extraction (DE) technology. Raw data were elaborated using the Data Explorer 5.0 software furnished by the manufacturer. The software package, Protein Prospector MS-Fit, available on-line at the web site of the University of San Francisco (CA, USA) (http://prospector.ucsf.edu), was used to identify protein spots from NCBI protein and gene sequence databases. A number of top candidates with high scores from the peptide matching analysis were further evaluated by comparing their calculated pI and MW using the experimental values obtained from 2-DE gel. These two parameters were used with large tolerances (DpI = 1 and DMW = ±15% Mw) as filters to exclude false positive candidates from the output lists.

2.5. Enzymatic activities

Enzymatic activities were determined in samples (150–300 μg of proteins) of cell-free extracts prepared by sonication (five 30 s pulses with intermitted 1 min cooling periods) in lysis buffer A (50 mM Tris–HCl pH 8, 100 mM NaCl,). For β-ketothiolase assay, the lysis buffer was 100 mM Tris–HCl, pH 7.3, containing 1 mM DTT. The cellular debris were removed by centrifugation at $12\,500 \times g$ for 60 min. β-Ketothiolase, and acetoacetyl-CoA reductase activities were determined according to the published methods [18]. Isocitrate dehydrogenase, and succinate dehydrogenase activities were assayed as described in Ref. [19,20], respectively. Aconitase activity was tested as described in Ref. [21], by using cell-free extracts anaerobically prepared. Protein concentration was determined by the Bradford assay [22] using Bovine Serum Albumin (BSA) as standard.

3. Results

3.1. Comparative analysis of 2-DE protein profiles and identification of selected protein spots

The availability of anti-RhdA antibodies that did not show cross-reactions with other two-domains rhodanese-like proteins [16] allowed the identification of *A. vinelandii* MV474 as a null RhdA strain. Western blot analysis clearly showed that no protein corresponding to RhdA was expressed in this mutant (see enlargement of Fig. 1), thus demonstrating that the residual detectable thiosulfate:cyanide sulfurtransferase activity of MV474 (20% as compared to that of UW136 strain) was not inferred by the protein coded by *rhdA*. ImageMaster spot matching software, along with RhdA spot identification in UW136 by Western-blotting, were used to check reproducibility of separations, and to compare the number of spots from averaged gels. Approximately 290 protein spots were detected on each gel image after Coomassie Blue staining of the gels, and, though the matching of the protein profiles of *A. vinelandii* UW136 and MV474 revealed changes in intensity of several protein spots, only spots significantly altered in the Coomassie Blue-stained protein profiles of the mutant strain were considered. MALDI-TOF analysis was at first restricted to the spots 1, 2, and 3 of Fig. 1. The protein spot number 3, present only in the mutant, was identified as kanamicyn kinase that was used as a selection marker for construction of the *rhdA A. vinelandii* mutant [9]. The spots 1 and 2 were identified in both strains as the products of the *phbB* and *phbA* genes, present in the PHB biosynthetic operon [14], acetoacetyl-CoA reductase, and β-ketothiolase, respectively. As shown in Table 1, the proteins acetoacetyl-CoA reductase, and β-ketothiolase were upregulated in MV474, when compared with the wild-type strain.

3.2. Phenotype of the A. vinelandii *rhdA* mutant

Comparable growth rates were observed for both *A. vinelandii* UW136 and *rhdA* mutant strains aerobically grown in BSN medium in the presence of sucrose as carbon source (data not shown). To verify whether the up-regulation of the product of the *phbB* and *phbA* genes in the *rhdA* mutant could result in PHB accumulation, quantification of PHB in both *A. vinelandii* strains, and ultrastructural analysis of the cells were performed. PHB content (determined in three separated growth experiments) was 50 ± 5 μg/mg protein in UW136, and

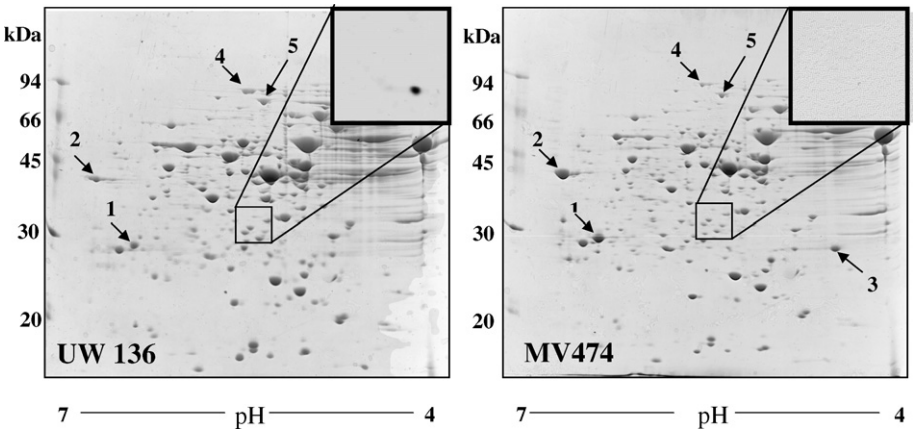

Fig. 1. Coomassie blue-stained protein profiles of *A. vineladii* wild-type (UW136) and *rhdA* mutant (MV474) strains after 25 h growth in BSN medium supplemented with 1% sucrose. Numbers represent the spots identified by MALDI-TOF MS analysis listed in Table 1. Enlargements display Western blot analysis of the region of RhdA spot.

Table 1
Proteins from *A. vinelandii* identified by peptide mass fingerprinting

Spot number	Protein name	Accession number[a]	Sequence coverage (%)	M_r/p*I*	Fold increase[b]
1	Acetoacetyl-CoA reductase	Q93QF0	63	26726 kDa/6.2	2.08
2	β-Ketothiolase	Q9KH97	50	40875 kDa/6.7	5.49
3	Kanamycin kinase	P00552	75	30961 kDa/5.1	Absent in UW136
4	Aconitate hydratase 2	Q4J5V0	22	93347 kDa/5.5	1.02
5	Aconitate hydratase 1	Q4IW32	25	97314 kDa/5.3	0.92

[a]Swiss-Prot/TrEMBL database.
[b]%*V* relative abundance in MV 474 vs UW136.

330 ± 10 μg/mg protein in the mutant strain, indicating that the metabolic profile of the mutant MV474 did favor synthesis and accumulation of this polymer. Furthermore, electron microscopy analyses (Fig. 2), revealed that the cells of the mutant strain contained numerous electron transparent globules, rather uniform in shape and size (300 × 200 nm). Similar globules were also present in the UW136, though in a very limited number, and with a much smaller size. No other differences in the ultrastructural details were appreciable between the two *A. vinelandii* strains.

The activity of the enzymes acetoacetyl-CoA reductase and β-ketothiolase involved in PHB synthesis was, therefore, tested in the cell extracts from both strains at the same time growth (25 h) of the 2-DE analyses (Table 2). In agreement with protein upregulation in MV474 (Table 1), both activities were found significantly higher in the *A. vinelandii* mutant than in the wild-type strain.

In *A. vinelandii*, synthesis of PHB has been related to the functioning of the tricarboxylic acid (TCA) cycle, since under balanced growth conditions acetyl-CoA is mainly fed into TCA cycle [18,23]. To assess whether RhdA deficiency could affect the activity of enzymes of the TCA cycle, succinate dehydrogenase, aconitase and isocitrate dehydrogenase activities were tested (Table 2). The activity figures of isocitrate dehydrogenase were identical in both strains, whereas in the *rhdA* mutant succinate dehydrogenase activity was about 60% of that measured in the *A. vinelandii* wild-type strain, and aconitase activity was not detectable. Noticeably, the TCA enzymes

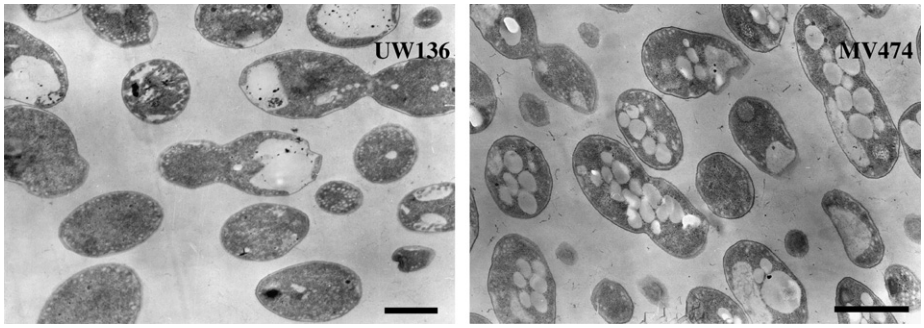

Fig. 2. Electron micrography (magnification = ×30000) of the strains UW136 and MV474 after 25 h growth (OD_{600} = 2.80) in BSN supplemented with 1% sucrose. Bars = 1 μm.

Table 2
Enzymatic activities

Strain	Enzyme				
	β-Ketothiolase[a]	Acetoacetyl-CoA reductase[b]	Succinate dehydrogenase[c]	Isocitrate dehydrogenase[d]	Aconitase[e]
UW 136	29.8 ± 0.6	1.02 ± 0.13	0.41 ± 0.06	0.038 ± 0.003	2.45 ± 0.07
MV 474	445 ± 34	2.10 ± 0.25	0.24 ± 0.09	0.038 ± 0.003	n.d.

n.d.: not detectable.
[a]nmol acetyl-CoA formed min^{-1} mg $protein^{-1}$.
[b]μmol $NADP^+$ formed min^{-1} mg $protein^{-1}$.
[c]μmol reduced DCIP formed min^{-1} mg $protein^{-1}$.
[d]μmol NADH formed min^{-1} mg $protein^{-1}$.
[e]μmol *cis*-aconitate formed min^{-1} mg $protein^{-1}$.

affected by RhdA deficiency are Fe–S proteins, and on aconitase, that contains a very labile redox center [24], the effect was dramatic.

3.3. Further characterization of the A. vinelandii null RhdA strain

The dramatic effect inferred by RhdA deficiency on the activity of aconitase prompted us to analyze the expression of aconitase polypeptides in both strains. The spots number 4 and 5 of Fig. 1 were subjected to MALDI-TOF analysis, and they were identified as Q4J5V0_AZOVI (aconitate hydratase 2) and Q4IW32_AZOVI (aconitate hydratase 1), respectively. The relative abundance of these spots (%V), was accurately calculated from independent gel patterns for each set (UW 136 and RhdA null mutant *A. vinelandii* strains), and, as shown in Table 1, expression of aconitate hydratase 1 and 2 was comparable in both strains. A "metabolic profile" similar to that caused by RhdA deficiency was also depicted as a consequence of *cydR* inactivation [25]. CydR is an Fnr-like protein exceptionally oxygen-labile, and transcriptionally inactive CydR can be converted into the active form by enzymatic reconstitution (in the presence of the cysteine desulfurase NifS) of its Fe–S cluster [26]. The vulnerability of some Fe–S clusters elicited the problem of Fe–S cluster protection in aerobic organisms [27,28], and the results of our investigation could be taken as an indication that RhdA deficiency avoided triggering some protection in the aerobe *A. vinelandii*. To approach this issue, we investigated the impact of RhdA deficiency in conditions mimicking oxidative stress (i.e. the addition of the oxidative agent PMS). As shown in Fig. 3, the resistance of *A. vinelandii* to oxidative stress was significantly impaired in the absence of RhdA. In the case of the *rhdA* mutant, the OD_{600} recorded after PMS treatment was about 40% of that of untreated control cultures, whereas the same OD_{600} was recorded for the wild-type strain, no matter PMS exposure.

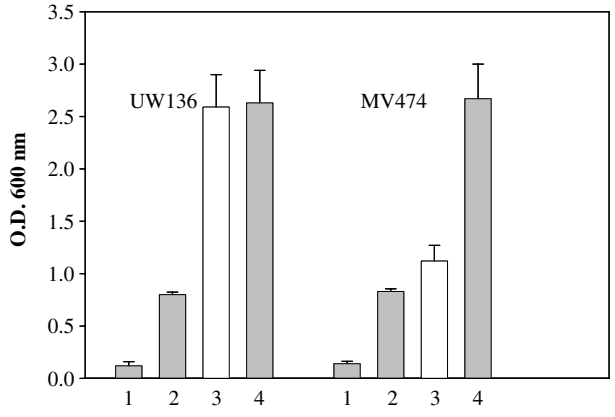

Fig. 3. Effect of phenazine methosulfate (PMS) on growth of *A.vineladii* wild-type (UW136) and *rhdA* mutant (MV474) strains. Growth of UW 136 and MV474 strains in BSN medium supplemented with 1% sucrose was estimated by measuring OD_{600} at the time 0 h (referred as 1), and 8 h (referred as 2). At this time, both cultures (UW136 and MV474) were divided into two samples, one was treated with PMS (final concentration 15 μM), and the other was not treated. The OD_{600} recorded after 16 h exposure to PMS, and that of untreated control cultures are referred as 3 and 4, respectively.

4. Discussion

The difficulties in establishing *in vivo* functions of rhodaneses lie in the redundancy of rhodanese-like proteins in the organisms. In this study, the *A. vinelandii* mutant MV474 was identified as an RhdA null strain despite the presence of residual thiosulfate:cyanide sulfurtransferase activity due to the multiple rhodanese-like proteins in this organism (Accession number PF00581, *Azotobacter vinelandii AvOP*). No difference in growth rate and in ultrastructural cell details were appreciable between the *A. vinelandii* wild-type UW136 and the *rhdA* mutant strains, but the mutant accumulated PHB when cells were aerobically grown to early stationary phase. In many bacterial species, accumulation of PHB in the stationary phase of growth, as cytoplasmatic hydrophobic granules, represents a reserve of carbon and energy [29]. The observation that at the onset of stationary phase, both *rhdA* mutant and the wild-type strains contained similar electron transparent PHB globules (data not shown), indicated that RhdA deficiency should trigger "mechanisms" for early production of PHB. Production of PHB in the *A. vinelandii rhdA* mutant was driven by upregulation of the *phbA* and *phbB* gene products β-ketothiolase and acetoacetyl-CoA reductase [14]. Acetyl-CoA utilization in the route of PHB synthesis was also favoured by the decrease of activity of the TCA enzymes succinate dehydrogenase and aconitase. The effect of RhdA deficiency was dramatic on aconitase activity, in spite of comparable expression of aconitase polypeptides in both strains. These findings indicated that in the *rhdA* mutant the null enzymatic efficiency was inferred by "inactivation" of aconitase enzymes. It has been reported that Fe–S cluster of aconitase is highly sensitive to oxidants, whereas succinate dehydrogenase Fe–S clusters are more stable [24].

The interplay between low TCA cycle activity and PHB accumulation is recognized, since under balanced growth conditions acetyl-CoA is mainly fed into TCA cycle [18,23]. As for the transcriptional regulation, in *A. vinelandii* the PHB biosynthetic gene cluster (*phbBAC*), coding for the enzymes of PHB synthesis, was found to be dependent on the transcriptional activator PhbR (a member of the AraC family), and in the proposed model PhbR activates the transcription of *phbB* [14]. Overexpression of β-ketothiolase and acetoacetyl-CoA reductase was also found as a consequence of *cydR* inactivation [25], and the control by CydR of PHB synthesis has been related to the presence of a putative CydR binding site within the *phbB* promoter region [14]. CydR is an oxygen responsive, DNA binding, Fnr-like protein that is exceptionally oxygen-labile [26]. Although mechanistic details of the effect of *rhdA* inactivation on CydR remain to be determined, it seems conceivable that the overexpression of the *phbA* and *phbB* gene products in the RhdA null mutant should be related to the CydR integrity. In support to the notion that RhdA could have a role in preserving labile Fe–S from aerobic damage, was the observation that the resistance of *A. vinelandii* to oxidative stress (i.e. PMS treatment) was significantly impaired only in the mutant strain. Our results corroborate the hypothesis that some of the abundant rhodanese-like proteins have roles in "managing" stress tolerance and in maintenance of redox homeostasis [30–33].

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